

092523

Throughout this application, various publications are cited. The disclosure of these publications is hereby incorporated by reference into this application to describe more fully the state of the art to which this invention pertains.

This invention relates to a positive selection method for isolating CD8⁺ cells using certain CD8-specific antibodies. The isolated CD8⁺ cells have importance as vehicles for combating viral infections and tumors.

20

In the past, purification of human CD8⁺ cells has
35 been achieved by negative selection. Specifically,

008020 2257550

peripheral blood mononuclear cells ("PBMC's") are
incubated with a cocktail of monoclonal antibodies
specific for non-CD8 sub-populations. These sub-
populations include, for example, B-cells, CD4⁺ cells, NK
5 cells, macrophages and neutrophils, and each contains
specific, non-CD8 "markers". The sub-populations are then
removed from the resulting antibody cocktail using
magnetic beads [4]. This technique has certain major
disadvantages. The first is that several monoclonal
10 antibodies are required for removing non-CD8⁺ cells. The
second is that the resulting CD8⁺ population suffers from
contamination from non-CD8⁺ cells that possess relatively
low levels of non-CD8 markers. Finally, when a magnetic
separation procedure is used to remove all non-CD8⁺ cells,
15 a large number of magnetic beads are needed.

Summary of the Invention

This invention provides a method of isolating CD8⁺ cells which comprises the steps of

- a
- 5 (a) contacting a sample of isolated peripheral mononuclear blood cells with a first antibody which specifically binds to the sequence AAEGLDTRFSG, (SEQ ID NO:1) or portion thereof, on CD8 molecules present on the surface of CD8⁺ cells
- 10 but does not activate the CD8⁺ cells once bound thereto, under conditions permitting the formation of a first complex between the CD8⁺ cell and first antibody;
- 15 (b) separating from the sample any first antibody not present in the resulting first complex;
- 20 (c) contacting the sample with a second, immobilized antibody which specifically binds to the first antibody in the first complex, under conditions permitting the formation of an immobilized, second complex between the first complex and the second antibody, thereby immobilizing the CD8⁺ cells present in the sample;
- 25 (d) separating from the resulting immobilized second complex the cells present in the sample which were not immobilized in step (c);
- 30 (e) contacting the immobilized second complex under suitable conditions with an agent which causes the dissociation of the second complex into CD8⁺ cells and an immobilized third complex between the first antibody and second antibody; and
- (f) separating the immobilized third complex from the CD8⁺ cells, thereby isolating the CD8⁺ cells.

This invention also provides a hybridoma cell line which produces a monoclonal antibody which specifically binds to CD8 molecules present on the surface of CD8⁺ cells but does not activate the CD8⁺ cells. This
5 invention further provides monoclonal antibodies produced by each of the instant hybridoma cell lines. Finally, this invention provides related polypeptides, isolated CD8⁺ cells and kits.

09561527 030800

Detailed Description of the Invention

Sub B2
5 The hybridoma cell lines designated 37B1 and 8G6 were deposited pursuant to, and in satisfaction of, the requirements of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, Virginia 2010-2209 under ATCC Accession Nos. HB-12441 and 10 HB-12657, respectively.

00521527.030800
This invention provides a method of isolating CD8⁺ cells by employing an anti-CD8 antibody, along with certain other reagents. Specifically, this invention 15 provides a method of isolating CD8⁺ cells which comprises the steps of

- (a) contacting a sample of isolated peripheral mononuclear blood cells with a first antibody which specifically binds to the sequence AAEGLDTQRFSG, (SEQ ID NO:1) or portion thereof, on CD8 molecules present on the surface of CD8⁺ cells but does not activate the CD8⁺ cells once bound thereto, under conditions permitting the formation of a first complex between the CD8⁺ cell and first antibody; 20
- (b) separating from the sample any first antibody not present in the resulting first complex;
- (c) contacting the sample with a second, immobilized antibody which specifically binds to the first antibody in the first complex, under conditions permitting the formation of an immobilized, second complex between the first complex and the second antibody, thereby immobilizing the CD8⁺ cells present in the sample; 25 30

- (d) separating from the resulting immobilized second complex the cells present in the sample which were not immobilized in step (c);
- 5 (e) contacting the immobilized second complex under suitable conditions with an agent which causes the dissociation of the second complex into CD8⁺ cells and an immobilized third complex between the first antibody and second antibody; and
- 10 (f) separating the immobilized third complex from the CD8⁺ cells, thereby isolating the CD8⁺ cells.

As used herein, a "CD8⁺ cell" means a T-cell having on its surface the CD8 protein. In the preferred embodiment, the CD8⁺ cells are human CD8⁺ cells. The CD8⁺ cells can be from any CD8⁺ cell-possessing species. "Isolating" CD8⁺ cells means obtaining a population of peripheral mononuclear blood cells wherein the ratio of CD8⁺ cells to non-CD8⁺ cells is at least about 7:1. In 20 the preferred embodiment of this invention, this ratio is at least about 9:1.

Sub
01 This invention employs several types of antibodies which specifically bind to given epitopes. More particularly, this invention uses a "first antibody" which 25 specifically binds to the sequence AAEGLDTQRFSG, or portion thereof, on CD8 molecules present on the surface of CD8⁺ cells but does not activate the CD8⁺ cells once bound thereto. Here, CD8⁺ cell "activation" means causing 30 CD8⁺ cells to express γ -interferon (" γ -IFN"). This activation can be measured using routine methods such as sandwich ELISA assays, which can be performed using commercially available kits.

portion of the constant (Fc) region of the first antibody. Such anti-Fc antibodies are commercially available and include, for example, sheep anti-mouse antibody immobilized on magnetic beads [5].

5
Sub
C2

The agent that causes dissociation of the immobilized second complex into CD8⁺ cells and immobilized antibodies can be any agent which successfully competes with the CD8 molecule for specific binding to the first antibody. In
10 the preferred embodiment, this agent is the polypeptide designated CD8-3 having the sequence AAEGLDTQRFSG. In one embodiment, the immobilized second antibody comprises an antibody operably affixed to a magnetic bead.

15 This invention also provides a hybridoma cell line which produces a monoclonal antibody which specifically binds to CD8 molecules present on the surface of CD8⁺ cells but does not activate the CD8⁺ cells. In one embodiment, the hybridoma cell line is selected from the
20 cell lines designated 37B1 (ATCC Accession No. HB-12441) and 8G6 (ATCC Accession No. HB-12657). This invention further provides the monoclonal antibodies produced by each of the instant hybridoma cell lines.

Sub
25
C3

This invention further provides a polypeptide useful for generating the instant monoclonal antibody that comprises the amino acid sequence AAEGLDTQRFSG. In the preferred embodiment, the polypeptide is the polypeptide designated CD8-3 and having the amino acid sequence
30 AAEGLDTQRFS. The instant polypeptide can optionally comprise one or more additional amino acid residues at the C-terminal or N-terminal end. In the preferred embodiment, the polypeptide has the sequence NKPKAAEGLDTQRFSGKRLG.

008020" 22572560

This invention further provides a population of CD8⁺ cells isolated by the instant method.

Sub 5
C4

Finally, this invention provides a kit for use in isolating CD8⁺ cells which comprises, in separate compartments, (a) an antibody which specifically binds to the sequence AAEGLDTRFSG, or portion thereof, on CD8 molecules present on the surface of CD8⁺ cells, but does not activate the CD8⁺ cells once bound thereto; and (b) an agent which causes the dissociation of a CD8⁺ cell-antibody complex. In one embodiment, the agent which causes the dissociation of a CD8⁺ cell-antibody complex comprises the polypeptide having the sequence AAEGLDTRFSG. In the preferred embodiment, the agent is the polypeptide consisting of the sequence AAEGLDTRFSG.

The instant kit can further comprise reagents useful for performing the binding and dissociation steps of the instant method. The components of the instant kit can either be obtained commercially or made according to well known methods in the art. In addition, the components of the instant kit can be in solution or lyophilized as appropriate. In the preferred embodiment, the kit further comprises instructions for use.

The following procedures relating to the instant invention are routine in the art: isolating peripheral mononuclear blood cells from which the CD8⁺ cells are in turn isolated [6]; separating unbound antibodies and cells from a sample containing bound antibodies and/or cells via centrifugation or spinning membrane; and immobilizing antibodies via polystyrene flasks, columns or beads [4,7].

This invention will be better understood by reference to the Experimental Details which follow, but those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative of the invention
5 as described more fully in the claims which follow thereafter.

008050" 4252560

Experimental Details

Rationale

5 Human CD8⁺ cells can be isolated from preparations of
peripheral blood mononuclear cells (PBMC's) by either
positive or negative selection. Positive selection
results in a highly-purified population of CD8⁺ cells.
Negative selection, while resulting in sufficient numbers
10 of CD8⁺ cells, has low levels of contaminating non-CD8
populations remaining after the selection procedure.

The idea was to generate an antibody which has high
affinity for CD8⁺ cells, does not activate the cells
15 during the selection process, and is capable of being
easily eluted from the cells. An anti-peptide antibody
appeared to meet these criteria. However, it was known
that anti-peptide antibodies might be of low affinity and
may recognize the linear peptide sequence exclusively,
20 preventing reactivity with native antigen.

It was necessary that the anti-CD8 antibody not
activate the cells during the selection process, as it
would lessen their ability to effectively act as naïve
25 responder cells during *in vitro* stimulation protocols.
The use of peptide release to selectively isolate a cell
population has been shown by Tseng-Law, et al. [8] for
CD34⁺ cells.

008020 257550

Methods

The CD8 alpha chain was examined for hydrophilic sequences and four peptides selected. All were coupled to keyhole limpet hemocyanin (KLH) as carrier and used to immunize mice. A C-terminal amino acid was added to each of the peptides coupled to KLH to make the monoclonal antibodies. Antisera from the mice were evaluated for the ability to recognize both peptide and native CD8 on the surface of T cells. Only one of the four peptides was capable of recognition of both antigenic forms of CD8. Monoclonal antibodies were generated to this peptide and the resulting antibody used to isolate CD8⁺ cells from a PBMC preparation. The antibody was successful in isolating a population of highly-purified CD8⁺ cells (Table 1) which were not activated by the isolation procedure (Table 2).

5

Table 1
Purification of CD8⁺ Cells by
Positive Selection Analyzed by Flow Cytometry*

CELL TYPE	PEMC Fluorescence (range)	CD8 ⁺ Cells Fluorescence (range)
CD8 T cells	15 (7-24)	82 (56-95)
CD4 T cells	36 (14-52)	2 (0.1-10)
CD14 Monocytes	15 (7-26)	0.8 (0.2-2)
CD15 Neutrophils	12 (8-21)	0.6 (0.1-3)
CD19 B cells	2 (0.4-7)	3 (0.5-9)
CD56 NK cells	6 (2-17)	6 (0.1-20)

* Summary of 10 normal donors

10

Table 2
Activation of CD8⁺ Cells Isolated By Negative or
Positive Selection (Assessed by IFN γ Production)

Cells	Negative Selection (pg/ml)	Positive Selection (pg/ml)
un-stimulated	20	20
allo-stimulation	1440	3600

References

1. Nabholz M. and H.R. MacDonald (1983) Annual Review of Immunology 1:273-306.
5
2. Riddell S.R. and P.D. Greenberg (1994) Current Topics in Microbiology and Immunology 189:9-34.
3. Riddell S.R. and P.D. Greenberg (1995) Annual Review of Immunology 13:545-586.
10
4. Horgan K and S. Shaw (1994) Current Protocols in Immunology 2:7.4.1.
- 15 5. Lea T, et al. (1988) Journal of Molecular Recognition 1(1):9-18.
6. Kanof, M.E., et al. (1994) Current Protocols in Immunology 2:7.1.1.
20
7. Kanof M.E. (1994) Current Protocols in Immunology 2:7:3:1.
8. PCT International Publication No. WO 95/34817.
25

Ans B+